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28. We thank M. Fitzgerald-Hayes for providing the *CEN3* probes, J. Szostak for providing for the *TCM*^{S}*1* gene on plasmid pSZ414 and S. Jinks-Robertson for providing plasmid pSR7; Nina Brown for technical assistance; S. Picologlou, and D. Morrison for helpful comments about the manuscript; and W. O. Godtfredsen and Leo Pharmaceutical Products for the gift of trichodermin. Supported by NIH grant GM24110 from the National Institute of General Medical Sciences.

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A Ligase-Mediated Gene Detection Technique

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An assay for the presence of given DNA sequences has been developed, based on the ability of two oligonucleotides to anneal immediately adjacent to each other on a complementary target DNA molecule. The two oligonucleotides are then joined covalently by the action of a DNA ligase, provided that the nucleotides at the junction are correctly base-paired. Thus single nucleotide substitutions can be distinguished. This strategy permits the rapid and standardized identification of single-copy gene sequences in genomic DNA.

DNA ANALYSIS IS ATTAINING increasing importance for the diagnosis of disease caused by single-gene defects as well as for the detection of infectious organisms (*1*). Moreover, a number of genes, predominantly those encoded in the major histocompatibility complex, have been found to be associated with an increased susceptibility to a variety of disease states (*2*). Of a total of approximately 2000 defined human genetic loci (*3*), approximately 100 have currently been studied at the DNA level for their role in genetic disease (*4*). A number of genetic diseases are caused by alleles present in the population at relatively high frequencies, perhaps because of selective advantages to the heterozygous carriers (*5*). The ongoing characterization of disease-causing or disease-associated gene sequences makes large-scale screening for carrier status and genetic counseling a possibility. It may also sharpen the diagnostic accuracy for diseases such as autoimmune conditions where the susceptibility may be influenced by defined alleles. Such prospects are currently limited by the cumbersome nature of the available DNA detection methods.

A majority of polymorphisms in the human genome are caused by point mutations that involve one or a few nucleotides. Current DNA analysis procedures capable of detecting the substitution of a single nucleotide are based on differential denaturation of mismatched probes as in allele-specific oligonucleotide hybridization (*6*) or denaturing gradient gel electrophoresis (*7*). Alternatively, the sequence of interest can be investigated for polymorphisms that affect the recognition by a restriction enzyme (*8*) or that will allow ribonuclease A (RNase A) to cleave at mismatched nucleotides of an RNA probe hybridized to a target DNA molecule (*9*). Although denaturing gradient gel or RNase A can survey long stretches of DNA for mismatched nucleotides, they are estimated to detect only about half of all mutations that involve single nucleotides (*7*, *9*). Similarly, less than half of all point mutations give rise to gain or loss of a restriction enzyme cleavage site (*10*). The only existing technique capable of identifying any single nucleotide difference, short of DNA sequence analysis, is allele-specific oligonucleotide hybridization. This technique involves immobilizing separated (*6*) or enzymatically amplified (*11*) fragments of target DNA, hybridizing with oligonucleotide probes, and washing under carefully controlled conditions to discriminate single nucleotide mismatches.

We have devised a strategy that permits the facile distinction of known sequence variants differing by as little as a single nucleotide. The approach combines the ability of oligonucleotides to hybridize to the sequence of interest and the potential of a DNA-specific enzyme, T4 DNA ligase, to distinguish mismatched nucleotides in a DNA double helix (Fig. 1). Two oligonucleotide probes are permitted to hybridize to the denatured target DNA such that the 3′ end of one oligonucleotide is immediately adjacent to the 5′ end of the other. The ligase can then join the two juxtaposed oligonucleotides by the formation of a phosphodiester bond, provided that the nucleotides at the junction are correctly base-paired with the target strand. The ligation event thus positively identifies sequences complementary to the two oligonucleotides. A heterozygous sample is therefore scored as positive for both alleles. The joining of the oligonucleotides may be conveniently demonstrated, for instance, by labeling one of the oligonucleotides with biotin and the other one with ^{32}P. After the ligation reaction, the biotinylated oligonucleotides are allowed to bind to streptavidin immobilized on a solid support. Radioactive oligonucleotides that have become ligated to biotinylated oligonucleotides remain on the support after washing and are detected by autoradiography.

The gene encoding human β globin was selected as a model system to test the technique. There are two relatively frequent alleles, β^S and β^C, each differing from the normal allele, β^A, by a single nucleotide substitution in positions 2 and 1, respectively, of codon six (Figs. 2 and 3) (*12*). Subjects homozygous for the β^S allele suffer from sickle cell anemia. Moreover an increased risk of sudden death during exertion has been observed among individuals heterozygous for β^S (*13*).

The ligase-mediated gene detection procedure was used to distinguish β^A and β^S genes in equivalent amounts of DNA present in cells, in cloned DNA, and in genomic DNA (Fig. 2). One of two synthetic oligonucleotides (B131 or B132), specific for each of the alleles, was used in conjunction

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with another oligonucleotide (P133) hybridizing immediately 3′ to either of the other two oligonucleotides on the target DNA strand. All of the synthetic oligomers used in this study are 20 nucleotides long. The ability of T4 DNA ligase to join the variable, 3′ nucleotide of the allele-specific oligonucleotides to the 5′ terminus of the invariant oligonucleotide was assessed by capturing any ligated product on streptavidin-agarose beads. The beads were filtered and washed to remove unbound oligonucleotides, and then the filter with trapped beads was exposed to x-ray film. The 10^6 nucleated cells used for one assay were obtained from ~0.5 ml of blood. The cells were used in the assay without DNA purification, by first making the DNA accessible for the ligase-mediated analysis by sequential additions of a nonionic detergent (Triton X-100) and a protease (trypsin). The DNA was denatured with alkali and then soybean trypsin inhibitor was added to prevent proteolysis of the added ligase.

The described ligation reactions were performed at 37°C, ~25 K below the melting temperature of the hybridized oligonucleotides, permitting the use of standardized assay conditions independent of the particular sequence investigated. The observed specificity is a consequence of the requirement for the simultaneous hybridization of both oligonucleotides in a precisely juxtaposed position. Although both oligonucleotides are likely to hybridize to numerous sequences in the DNA sample, they are unlikely to do so in the appropriate head-to-tail fashion except where the proper target sequence is present. In addition, we have found that the ligation reaction requires that the two terminal nucleotides on either side of the junction of the two oligonucleotides be engaged in correct base-pairing. This requirement further suppresses incorrect ligation events.

To determine whether any type of single nucleotide mismatch could be distinguished from correct base-pairing with the present method, we used four synthetic target molecules representing a segment of the β-globin gene, each with a different nucleotide in the first position of the sixth codon. Two of the sequences are derived from the β^A and β^C alleles of the β-globin gene. The other two sequences represent the other possible nucleotides occupying the variant position. Four pairs of oligonucleotides were designed to specifically identify one of the target molecules. Four oligonucleotide probes, each with a different nucleotide in the 3′ terminal position and complementary to one of the target molecules, were separately assayed for their ability to be ligated to an invariant oligonucleotide that hybrid-

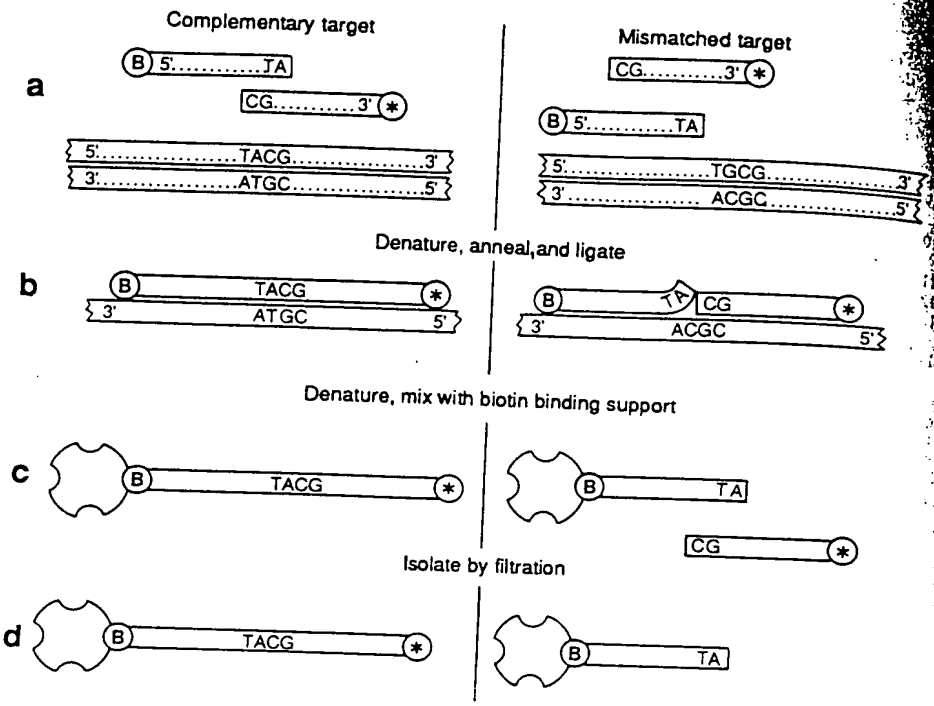

Fig. 1. A diagram depicting gene detection through the ligation of hybridized oligonucleotide probes. Target DNA is denatured and mixed with oligonucleotides and ligase. The ligase joins pairs of oligonucleotides annealed head to tail if they are correctly base-paired at the junction. Radioactively labeled oligonucleotides (*) are immobilized and detected by autoradiography only if ligated to biotinylated oligonucleotides (B) that can be bound to streptavidin on a solid support.

a

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                        MetValHisLeuThrProGl    uGluLysSerAlaValThr
B131  (βA)  5'  B  ATGGTGCACCTGACTCCTGA  pGGAGAAGTCTGCCGTTACTG  3' P133
B132  (βS)  5'  B  -------------------T
                                     Va   l
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b

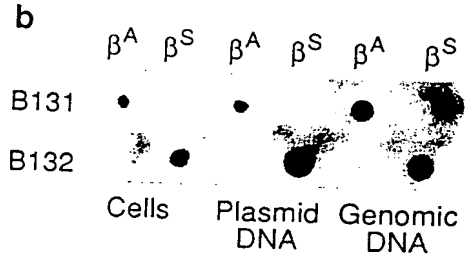

Fig. 2. (**a**) Nucleotide sequence and corresponding translated sequence of the oligonucleotides used in the analysis described in (b). (**b**) Analysis for the presence of the globin β^A or β^S allele in samples containing equal numbers of copies of β-globin alleles present in nucleated cells, in cloned DNA, and in genomic DNA. Two × 10^6 linearized plasmid molecules containing the β^A or β^S allele of the human globin genes were added to individual microtiter wells containing 10 μg of salmon sperm DNA in 4 μl of water (*19*). The microtiter plates were centrifuged and the supernatants removed. To the resuspended cell pellet was added 1 μl of 10% Triton X-100 and 1 μl of trypsin at 2 μg/μl. The samples were incubated at 37°C for 30 min and were denatured with alkali as above. The *p*H was neutralized and 1 μl of soybean trypsin inhibitor (Sigma, 10 μg/μl) was added. Each well received 140 fmol of biotinylated oligonucleotides B131 or B132 (*20*), specific for the globin β^A and β^S genes, respectively, and 1.4 fmol of oligonucleotide P133, 5′ end-labeled with [γ-^{32}P]adenosine triphosphate (ATP) and polynucleotide kinase to a specific activity of 5 × 10^8 Cerenkov cpm/μg and purified over a Nensorb column (Du Pont Biotechnology Systems). T4 DNA ligase (0.05 Weiss unit, Collaborative Research) was added in 2 μl of 5× ligase buffer to a final volume of 10 μl containing 50 m*M* tris-HCl (*p*H 7.5), 10 m*M* $MgCl_2$, 150 m*M* NaCl (including 50 m*M* added during denaturation), 1 m*M* spermidine, 1 m*M* ATP, 5 m*M* dithiothreitol, and 100 ng of bovine serum albumin per microliter. The reagents were mixed by briefly centrifuging the microtiter plates before incubating at 37°C and 100% humidity for 5 hours. The ligated oligonucleotides were denatured by the addition of 1 μl of 1.1*M* NaOH and incubated for 10 min at 37°C. After the incubation, 1 μl of 1.1*M* HCl and 2 μl of 10% SDS were added. Three microliters of a 15% (v/v) suspension of streptavidin-coated agarose beads (Bethesda Research Laboratories) was then added, and the plate was incubated on a shaking platform at room temperature for 5 min. The contents of the wells were transferred to a dot blot manifold (Schleicher and Schuell) with a Whatman filter paper no. 4. In order to reduce nonspecific binding of the labeled oligonucleotides, the filter papers had been boiled and the beads diluted in 0.5% (v/v) dry nonfat milk, 1% SDS, and salmon sperm DNA (100 μg/ml). The beads (*21*) were washed under suction in the manifold with 3 ml of 1% SDS and 1 ml of 0.1*M* NaOH per sample, with a 96-tip dispenser (Vaccu-pette/96, Culture Tek). The filters were wrapped in plastic wrap and autoradiographed for 3 days at −70°C with one enhancing screen (Du Pont).

immediately 3′ to the first oligonucleotide. These reagents permit studying the discrimination of ligation by any of the 16 possible base pairs, the 4 correct Watson-Crick pairs and 12 mismatched pairs, in an invariant sequence context. Under the appropriate conditions, only nucleotides engaged in correct base-pairing were efficiently joined by ligation (Fig. 3). Parameters that affected the nucleotide specificity were the salt concentration and the amount of enzyme added relative to the DNA concentration. Higher salt concentration and lesser amounts of enzyme than those found to be optimal for discrimination resulted in loss of signal. The above experiment cannot exclude the possibility that the identification of mismatched nucleotides may be influenced by the surrounding sequence, although we have not yet encountered any evidence for such effects.

Although autoradiographic techniques are relatively simple to implement, a gene detection assay based on the use of fluorescent rather than radioactive probes would have the advantages of safe handling, more stable reagents, and rapid access to the results, and would allow for multicolor analysis by using fluorophores with different emission spectra. In general, conventional organic fluorophores are less sensitive labels than ^{32}P. Thus we increased the amount of target DNA before the detection assay with the polymerase chain reaction (*14*). With this procedure a segment of DNA can be exponentially amplified by repeated cycles of enzymatic synthesis of new strands from two oligonucleotide primers, one with a sequence derived upstream and the other in the opposite orientation downstream of the segment of interest. Genomic DNA was obtained from three human cell lines, MOLT-4, which is homozygous for the β^A-globin allele; SC-1, homozygous for the β^S allele; and GM2064, in which the β-globin locus has been deleted (*15*). The appropriate segment of the β-globin gene was amplified in 25 cycles from 1 μg of genomic DNA from each cell line. We used 3-μl aliquots, equivalent to 24 ng of genomic DNA for the assay. Two oligonucleotides, specific for the β^A and β^S alleles and differentially 5′-labeled with one of two fluorophores, were present at equal concentrations. The amount of each of these oligonucleotides that became ligated to a third oligonucleotide hybridizing downstream of the other two was determined by separating the reaction products on an 8% polyacrylamide gel and analyzing the band migrating as a 40-nucleotide oligomer (the size of two ligated oligonucleotides) for the relative contribution by the two different fluorophores [model 370A DNA sequencer, Applied Biosystems, Foster City, California (*16*)]. No signal was observed when the β-globin gene had been deleted in the cell from which the DNA was obtained, whereas only the correct fluorophore-labeled oligonucleotide was ligated when the cells harbored the β^A or β^S alleles

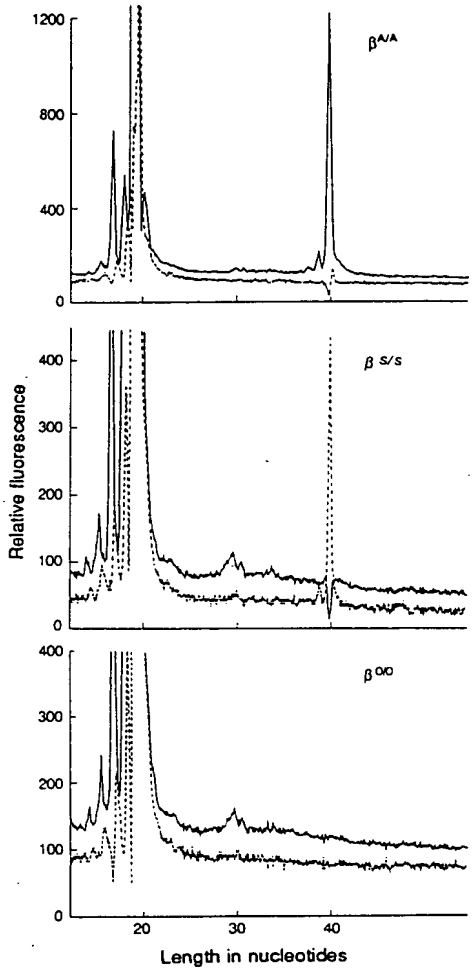

Fig. 4. Demonstration of the presence of the β^A and β^S alleles of the β-globin gene in amplified genomic DNA by probes labeled with fluorescent dyes. A 120-bp segment of the β-globin gene was amplified with the polymerase chain reaction as described (*16*) in 25 cycles starting with 1 μg of genomic DNA from the cell lines MOLT-4, SC-1, and GM2064 ($\beta^{A/A}$, $\beta^{S/S}$, and $\beta^{0/0}$, respectively) in 100 μl. Three microliters of each amplified sample was added to an Eppendorf tube, denatured by alkali, neutralized, and incubated with 14 fmol each of oligonucleotide 131 labeled with carboxy-fluorescein (Molecular Probes) (CF131) (——) and oligonucleotide 132 labeled with carboxy-2′,7′-dimethoxy-4′,6′-dichlorofluorescein (CD132) (– – – –), and 14 fmol of nonradioactively 5′ phosphorylated oligonucleotide P133 (for sequences, see Fig. 2). The reaction conditions were essentially as described in Fig. 2, but 0.5 Weiss unit of T4 DNA ligase was added to each assay. At the end of the 3-hour incubation, the samples were ethanol precipitated, taken up in 50% formamide, and loaded on a sequencing gel in an ABI 370A automated DNA sequencer. The fluorescence signal was processed to distinguish the partially overlapping emission spectra of the two fluorophores and to determine the relative contribution of each fluorophore to the signal.

a

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B128  (βA)  5' B  CATGGTGCACCTGACTCCTG  pAGGAGAAGTCTGCCGTTACT 3' P129
B134  (βC)  5' B  --------------------A
B136        5' B  --------------------T
B137        5' B  --------------------C

172   (βA)  3'    GTACCACGTGGACTGAGGACTCCTCTTCAGACGGCAATGA    5'
138   (βC)  3'    --------------------T--------------------   5'
139         3'    --------------------A--------------------   5'
140         3'    --------------------G--------------------   5'
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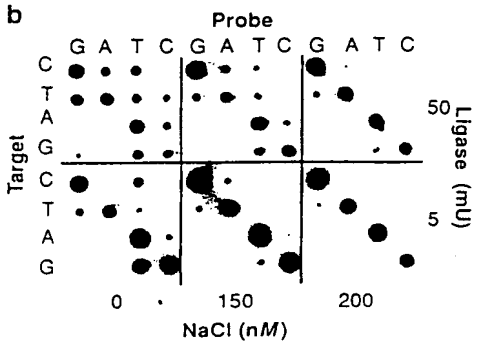

Fig. 3. (**a**) Nucleotide sequence of the oligonucleotides used in the analysis described in (**b**). (**b**) Correct identification of four target molecules, differing by single-nucleotide substitutions in one position. Letters refer to the variable nucleotides in the probe and target sequences. As target molecules, 40-nucleotide oligomers, derived from the β-globin gene sequence, were synthesized. The oligonucleotides 172, 138, 139, and 140 are of identical sequence except in a central position where each target molecule includes a different nucleotide. Four 20-nucleotide biotinylated oligomers, B128, B134, B136, and B137, differing only in their 3′ nucleotide position, were designed to hybridize to the 3′ half of the target molecules such that the variant position of the probe reagents corresponds to that of the target molecules. Each of the biotinylated oligonucleotides was used in conjunction with oligonucleotide P129, 5′ end-labeled with ^{32}P and hybridizing immediately 3′ to the biotinylated probes on the target strands. The assays were performed essentially as described in the legend to Fig. 2, but 2×10^8 copies of one of the target molecules were added to each well with 10 μg of salmon sperm DNA. Each well further received one of the biotinylated oligonucleotides together with oligonucleotide P129. The final NaCl and ligase concentrations were varied as indicated.

(Fig. 4). This strategy could be generalized to the simultaneous analysis of several loci. For each set of two labeled, allele-specific oligonucleotides and one unlabeled, the latter is given a nonhybridizing 3′ sequence extension of a unique length. This results in different migration rates for the ligation products, characteristic of each locus.

In contrast to gene detection techniques based on immobilizing the target DNA, such as DNA blots, the hybridization reported here was performed in solution and in a small volume, which reduced the time required for hybridization (*17*). It also obviated the step of immobilizing the target DNA. Both ligation and binding of the biotinylated oligonucleotides are efficient and rapid steps that should permit quantitative detection of target molecules. In general, there are three rate-limiting steps in gene detection techniques. The first is sample preparation, which can be greatly simplified as demonstrated here. The second is the time required for the probes to anneal to the target sequence. This is a function of the concentration of the probe and can be reduced considerably. The third and most time-consuming step in the present technique is signal detection by autoradiography. A sufficiently sensitive fluorescent detection method (*18*) should drastically reduce this time, permitting the development of a rapid, automated gene detection procedure.

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19. Genomic DNA was purified from guanidinium HCl–solubilized cells as described [D. Bowtell, *Anal. Biochem.* 162, 463 (1987)] and resuspended by boiling before adding 7 μg in 4 μl of water per assay well. The plasmid and genomic DNA samples were denatured by adding 1 μl of 0.5*M* NaOH and incubating for 10 min at 37°C before restoring the *p*H with 1 μl of 0.5*M* HCl. Alternatively, samples of nucleated blood cells were used directly as a source of DNA for the analysis. Cells (10^6), obtained by Ficoll-Hypaque (Pharmacia) flotation, were added in 50 μl of phosphate-buffered saline to each well.
20. The oligonucleotides were assembled by the phosphoramidite method [S. J. Horvath, J. R. Firca, T. Hunkapiller, M. W. Hunkapiller, L. Hood, *Methods Enzymol.* 154, 314 (1987)] on an Applied Biosystems model 380A DNA synthesizer and purified either by polyacrylamide gel electrophoresis or reversed-phase high-pressure liquid chromatography (HPLC). Biotinylation was performed by reacting a biotin *N*-hydroxysuccinimide ester (Enzotin, Enzo) with a 5′ aminothymidine residue incorporated in the oligonucleotide [L. M. Smith, S. Fung, T. J. Hunkapiller, M. W. Hunkapiller, L. Hood, *Nucleic Acids Res.* 13, 2399 (1985)]. The product was purified by reversed-phase HPLC.
21. The size of the area on which the beads were deposited was reduced by interposing a 3-mm-thick plexiglass disk with conical holes with diameters of 5 mm on the upper surface and 2 mm on the lower.

22. The authors acknowledge a stipend from the Knut and Alice Wallenberg Foundation to U.L. and support from NSF grant BNS 87 14486, Defense Advanced Research Projects Agency grant N00014-86K-0755, Upjohn Company, and Applied Biosystems, Inc. The oligonucleotides were synthesized by S. J. Horvath and the fluorescence data were analyzed by C. Dodd. R. K. Saiki provided plasmids and samples of genomic DNA obtained from cell lines. J. Korenberg and K. Tanaka made available blood samples from sickle cells patients. The *N*-hydroxysuccinimide ester of carboxy-2′,7′-dimethoxy-4′,5′-dichlorofluorescein was provided by M. W. Hunkapiller. We acknowledge discussions with B. Korber, B. Popko, A. Kamb, N. Lan, L. Smith, R. Barth, V. A. McKusick, J. Richards, and M. Simon.

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Amyloid Protein Precursor Messenger RNAs: Differential Expression in Alzheimer's Disease

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In situ hybridization was used to assess total amyloid protein precursor (APP) messenger RNA and the subset of APP mRNA containing the Kunitz protease inhibitor (KPI) insert in 11 Alzheimer's disease (AD) and 7 control brains. In AD, a significant twofold increase was observed in total APP mRNA in nucleus basalis and locus ceruleus neurons but not in hippocampal subicular neurons, neurons of the basis pontis, or occipital cortical neurons. The increase in total APP mRNA in locus ceruleus and nucleus basalis neurons was due exclusively to an increase in APP mRNA lacking the KPI domain. These findings suggest that increased production of APP lacking the KPI domain in nucleus basalis and locus ceruleus neurons may play an important role in the deposition of cerebral amyloid that occurs in AD.

ALZHEIMER'S DISEASE (AD) IS characterized pathologically by large numbers of senile plaques and neurofibrillary tangles throughout the cerebral cortex and hippocampus. Senile plaques consist of clusters of degenerating neurites surrounding an amyloid core composed of 5- to 10-nm fibrils that stain metachromatically with Congo red. In many cases of AD, amyloid fibrils are also found in vessel walls (*1*). A 4.2-kD polypeptide, referred to as A4 or the β protein, has been isolated from the amyloid fibrils found in senile plaques (*2*) and vessel walls (*3*) of patients with AD. There is evidence that A4 may also be a component of the paired helical filaments found in neurofibrillary tangles (*4*).

The gene encoding A4, which is located on chromosome 21 (*5*), produces at least three mRNAs (Fig. 1) referred to as APP_{695}, APP_{751}, and APP_{770} (*6–8*). APP_{695}, the mRNA that was initially identified (*5*), encodes an amyloid protein precursor (APP), 695 amino acids in length, that includes A4 at positions 597 to 638. APP_{751} is identical to APP_{695}, except for a 168-nucleotide insert (*6–8*). This insert, previously referred to as HL124i (*7*), would introduce 56 amino acids carboxyl terminal to Arg^{288} and convert Val^{289} into an isoleucine. APP_{770} is identical to APP_{751}, except for a 57-nucleo-

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